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The transmembrane domain and the proton channel in proton-pumping transhydrogenases

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Abstract

Proton-pumping nicotinamide nucleotide transhydrogenases are composed of three main domains, the NAD(H)-binding and NADP(H)-binding hydrophilic domains I (dI) and III (dIII), respectively, and the hydrophobic domain II (dII) containing the assumed proton channel. dII in the *Escherichia coli* enzyme has recently been characterised with regard to topology and a packing model of the helix bundle in dII is proposed. Extensive mutagenesis of conserved charged residues of this domain showed that important residues are βHis91 and βAsn222. The pH dependence of βH91D, as well as βH91C (unpublished), when compared to that of wild type shows that reduction of 3-acetylpyridine-NAD^+ by NADPH, i.e., the reverse reaction, is optimal at a pH essentially coinciding with the pK_a of the residue in the β91 position. It is therefore concluded that the wild-type transhydrogenase is regulated by the degree of protonation of βHis91. The mechanisms of the interactions between dI+dIII and dII are suggested to involve pronounced conformational changes in a 'hinge' region around βR265.

Keywords: Transhydrogenase; Proton pump; Membrane protein; Nicotinamide nucleotide; Nicotinamide adenine dinucleotide; Nicotinamide adenine dinucleotide phosphate

1. Introduction

Proton-translocating transhydrogenase (EC 1.6.1.1) is an integral membrane protein that is found in all organisms and cell types except yeasts, plants and certain bacteria. It is located in the cytoplasmic membrane of bacteria and in the inner membrane of mitochondria. The enzyme participates in the bioenergetic processes of the cell, and utilises the electrochemical proton gradient across the membrane to drive NADPH formation from NADH according to the reaction

$$H^+_{out} + NADH + NADP^+ \rightleftharpoons H^+_{in} + NAD^+ + NADPH \quad (1)$$

where 'out' and 'in' denote the cytosol and matrix, respectively, in mitochondria and periplasmic space and cytosol, respectively, in bacteria. As indicated by Eq. 1, transhydrogenase works as a proton pump in both directions. However, in the presence of an electrochemical proton gradient (Δp), the rate of the reaction from left to right is stimulated some 10-fold and the equilibrium is shifted towards NADPH formation from unity to about 500. The enzyme is ste-

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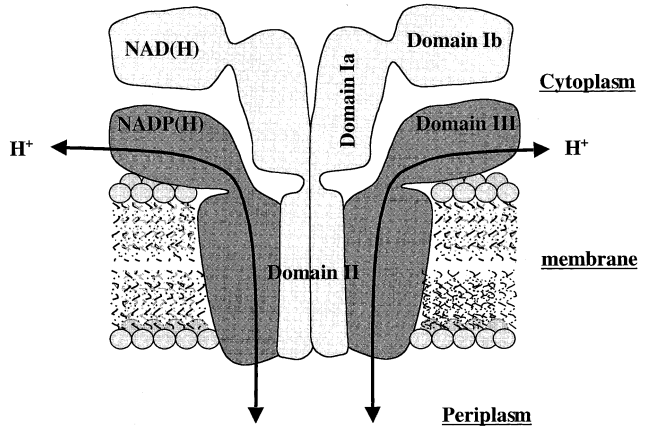

Fig. 1. A cartoon of the proton-translocating transhydrogenase from *E. coli*. Domain Ia and Ib refer to the subdomains of dI where domain Ib contains the NAD(H)-binding site.

reospecific for the 4A hydrogen of NADH and the 4B hydrogen of NADPH. It has been estimated that one proton is translocated across the membrane per net transfer of one hydride equivalent, i.e. the H^+/H^- is 1. Transhydrogenase also catalyses a non-proton-pumping cyclic reaction which involves binding of NADH, reduction of bound $NADP^+$ to NADPH, replacement of the NAD^+ formed by $AcPyAD^+$ and reduction of $AcPyAD^+$ by NADPH to AcPyADH.

The transhydrogenase-generated NADPH is essential for a high cellular/mitochondrial NADPH/$NADP^+$ ratio required for detoxication, regulation and maintenance of e.g. Ca^{2+} homeostasis and apoptosis. Purified transhydrogenases have been extensively characterised kinetically and reconstituted in liposomes with and without other proton pumps (for a review see [1,2]).

Transhydrogenase from *Escherichia coli* is composed of an α subunit of about 54 kDa and a β subunit of 48 kDa, with a native form of $\alpha_2\beta_2$ [3,4]. As all proton-translocating transhydrogenases, the *E. coli* enzyme is composed of three domains, i.e., the hydrophilic domain I (dI, residues α1–α402) containing the NAD(H)-binding site, the hydrophobic domain II (dII, residues α403–510 and β1–462) containing the membrane-spanning α helices, and the second hydrophilic domain III (dIII, residues β261–β462) containing the NADP(H)-binding site (Fig. 1). dII contains a single conserved and important protonatable residue, βHis91, which is believed to be directly involved in proton translocation [5–7]. The DNA coding for dI from e.g. *E. coli* [8,9], *Rhodospi-*

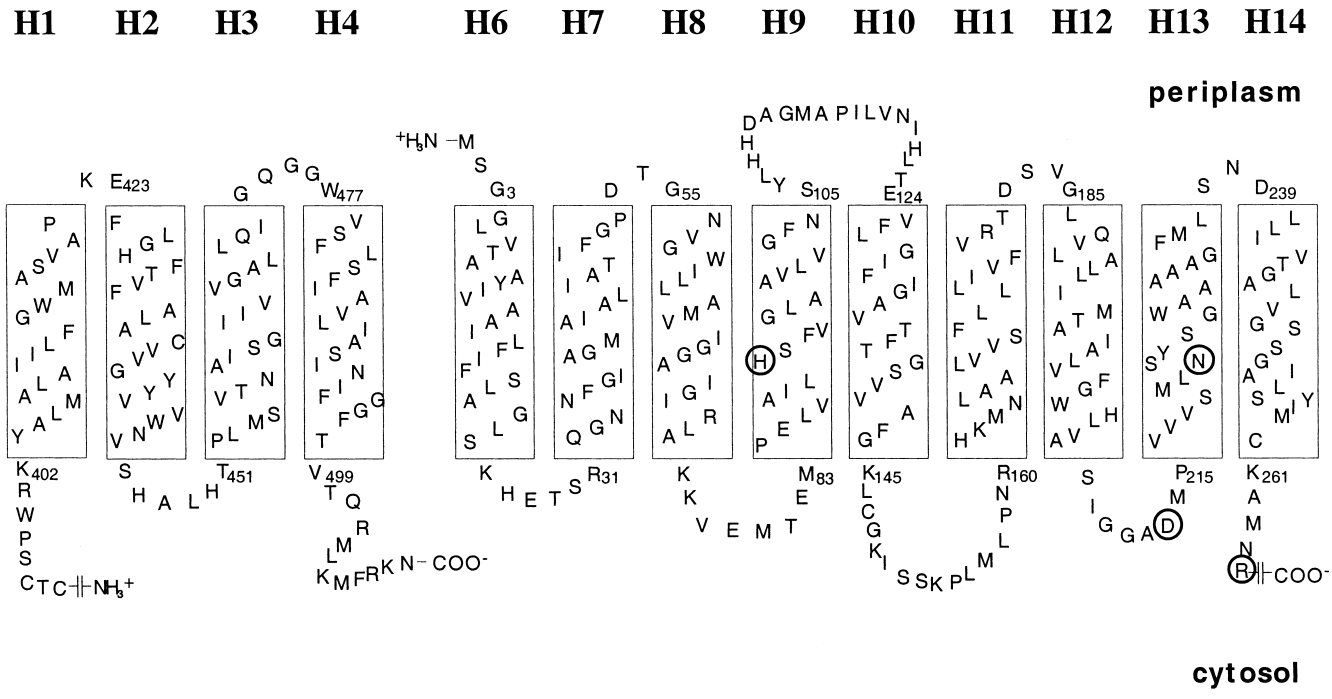

Fig. 2. The membrane topology of *E. coli* transhydrogenase. Encircled residues are the βHis91, βN222, βD213 and βR265.

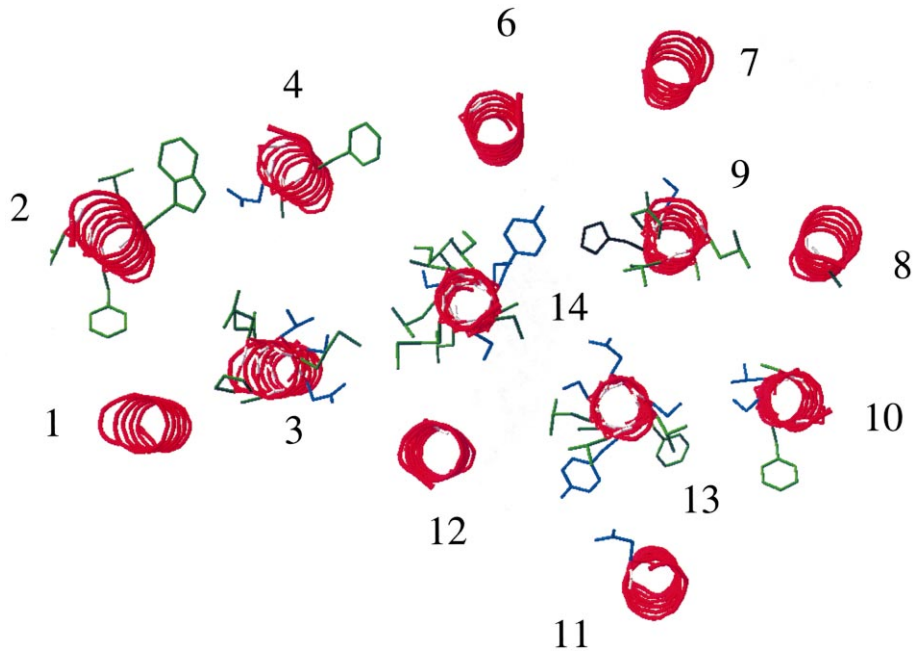

Fig. 3. Predicted helix packing of transhydrogenase domain II viewed from the cytoplasmic side. Residues conserved in at least 85% of the approx. 25 known transhydrogenase sequences are depicted, polar residues in blue, non-polar residues in green and basic residues in black. No conserved acidic residues are found in the transmembrane helices. Numbering of helices is taken from Fig. 2.

rillum rubrum [8] and the DNA coding for dIII from *E. coli* [9] and *R. rubrum* [10,11], bovine [12] and human [13] have been individually overexpressed and the proteins purified and characterised. Even in the absence of the transmembrane dII, a mixture of dI and dIII catalyses transhydrogenation although with markedly different kinetics. Thus, the dI+dIII system has provided an important tool for studying the hydride transfer partial reaction (cf. [14–16]). Recently, the folds of dIII have been deduced by NMR [17–19], and the structure solved by X-ray crystallography [20–22]. A cartoon of the *E. coli* transhydrogenase is shown in Fig. 1.

Despite the pronounced progress concerning the structure and function of dI+dIII, it is obvious that a breakthrough in the understanding of the mechanism of proton pumping cannot be achieved without more information about dII, the role and function of the proton channel, and the interactions between dII and dI+dIII. It has recently been argued that the proton channel in transhydrogenases does not have any important role but is simply a passive channel where proton flux is mediated by e.g. serine and threonine residues [21]. The purpose of this review is to demonstrate that key residues in the channel probably have fundamentally important functions similar to that of e.g. Asp61 in *E. coli* F_1F_0-ATPase [23].

2. Membrane topology of dII of *E. coli* transhydrogenase

The membrane topology and packing of the transmembrane α helices in dII are essential for the understanding of the function of dII in proton translocation and its interactions with dI and dIII. Until recently the topology of dII of the proton-translocating transhydrogenases based on predictions and various experimental approaches varied between 10 and 14 [24–26]. In a systematic study using a cysteine-free *E. coli* transhydrogenase, which has essentially wild-type properties, single cysteines were introduced in potential loops and their reactivities towards membrane impermeable thiol-specific maleimide reagents investigated [27]. The number of helices and their topological orientations were unambiguously determined for the entire membrane-spanning domain of

the protein, resulting in a 13-helix model where the C-terminus of the α subunit is localised on the cytosolic side and the N-terminus of the β subunit is localised on the periplasmic side of the membrane (Fig. 2).

The odd number of helices seemed first to be at variance with the topology of the mitochondrial enzyme. However, the additional 25 amino acid long peptide present in the single mitochondrial polypeptide was predicted as an extra helix. Because of the high sequence identity between the *E. coli* and bovine enzymes, the extra bovine helix could be viewed as linking the two *E. coli* subunits. This was in fact experimentally verified by fusing the α and β subunits of the *E. coli* enzyme with a peptide linker, which gave a close to wild-type enzyme, whereas a shorter linker gave a partly misfolded and inactive enzyme (J. Meuller, K. Mjörn, P.D. Bragg and J. Rydström, unpublished).

3. Helix packing in dII

The packing of the 26 α helices in the active dII dimer is unknown, but the very tight binding between the peptides in the *E. coli* dI dimer [9] suggests that the four helices of each α subunit are also closely located. An examination of conserved residues in the transmembrane helices reveal that helices 3, 9, 10, 13 and 14 possess more than 50% conserved residues (Fig. 3), suggesting that these form the basic structure for a functional helix bundle containing the proton channel. It should be stressed in this context that several helices may form part of the channel, i.e., the protons may jump from one helix to another. Interestingly, all these helices have a high content of Ala and Gly mixed with polar residues, which is a composition suitable for a water-filled channel formed by the above interconnecting helices. A cartoon of the intact *E. coli* transhydrogenase is shown in Fig. 4.

The properties of a set of deletion mutants demonstrated that helices 1–2 and 7–11 were not essential for assembly [4]. Subsequently, cross-linking studies showed that the two subunits, in addition to other potential interaction surfaces, are closely associated through residues αC395 and αC397 [28]. In addition, the α and β subunits in the intact *E. coli* enzyme are

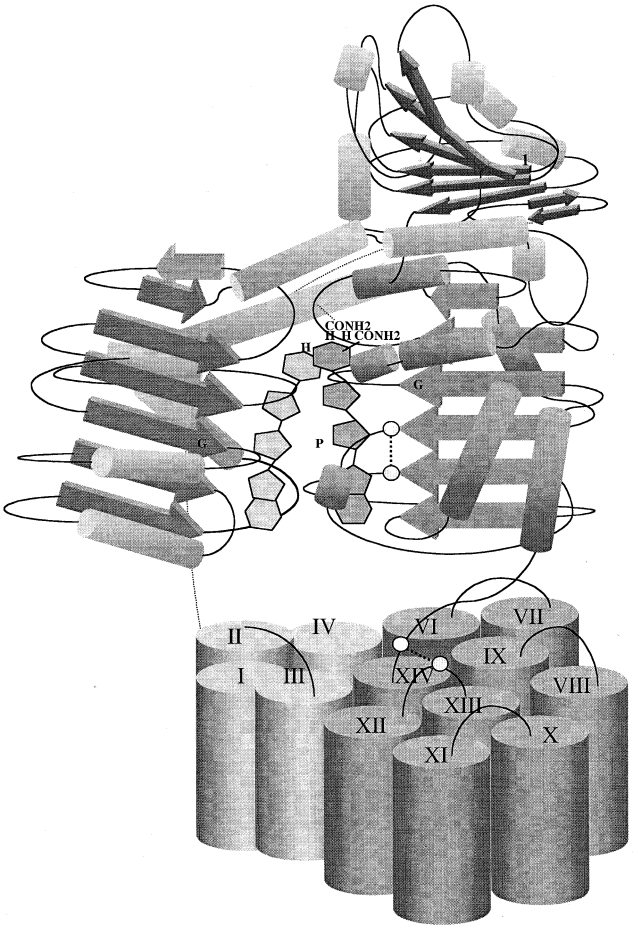

Fig. 4. A cartoon of the intact *E. coli* transhydrogenase, demonstrating the relative positions of dI, dIII and the helix bundle in dII. For clarity the dI+dIII parts have been lifted relative to dII. Dotted lines between small circles indicate possible salt bridges.

tightly bound through hydrophobic interactions between the transmembrane helices, where the β subunits probably do not interact but are associated with separate sides of the α subunit dimer [29]. In addition, dI is surprisingly flexible relative to dIII [29].

4. Conserved residues in dII important for proton translocation

In general, the β subunit is believed to contain the proton channel. The reason is that no conserved and functionally important residues have been found in the α subunit, which does not mean that various helical surfaces of the α subunit do not contribute

to the proton channel [30]. βHis91 was early shown to be important for proton pumping and catalytic activity [5–7], and mutations of βN222 located in helix 13 (cf. Fig. 2) were demonstrated to have drastic effects on all activities [31]. Residue βD213, which is located in a very conserved cytosolic loop, has also been suggested to be involved in proton translocation [5,12]. This suggestion was subsequently supported in a thorough and systematic investigation in which all conserved charged residues in the β subunit were replaced by mutagenesis [7].

A significant contribution to the understanding of the role of βHis91 was made by mutating it to βH91K [6,32] and by deleting the six last residues in the C-terminus of the α subunit of *E. coli* transhydrogenase [32]. In both cases, the affinity for NADP(H) increased markedly to a level similar to that in isolated dIII, suggesting that in the former case a constant positive charge had been introduced in dII resembling a protonated βHis91. In the latter case, a truncation of the proton channel is likely that led to a change of the properties of dIII similar to those typical for removal of dII.

A characterisation of the pH dependence of the reverse reaction catalysed by βH91E showed that the pH optimum, which in the wild-type enzyme is about 7.0, was shifted to approx. 5 [7]. Recently, the βH91C mutant was also found to be quite active, and showed a pH dependence with an optimum of 8 (T. Bizouarn, J. Meuller and J. Rydström, unpublished). In all these mutants, the pH optimum was thus essentially determined by the residue in the 91 position, i.e., 7 for His in wild type, 5 for Glu in βH91E, and 8 for Cys in βH91C. These results, together with the fact that the pH dependence of the reverse reaction catalysed by dI+dIII is essentially flat [16], *suggests that the reverse reaction is governed by the protonation state of βHis91*. Moreover, this conclusion strongly suggests that βHis91 is directly involved in all 'energy-linked', i.e. proton pumping-related, functions of the enzyme.

Substitution of the conserved βHis91 residue by an asparagine in four strains of *Mycobacterium* and in one *Porphyromonas* strain has led to the suggestion that βHis91 would only play a passive role [21]. The proton translocating part of domain II would thus be composed of a passive channel of polar side chains and bound water with all components for the coupling located outside the membrane, presumably in domain III [21]. This latter suggestion does not, however, account for the experimental results summarised above.

Examination of the transhydrogenase sequences of the *Mycobacteria* strains might provide an explanation for the varying residue in position β91. Several other highly conserved residues, e.g., in the indicated helices 3, 9, 13 and 14, are substituted with a strong preference for the substitutions to be located on one side of the helix. Especially notable is the appearance of a histidine residue, at approximately the same depth as βH91C, in helix 3. Thus, the nature of the substitutions in the *Mycobacterium* strains indicates that compensatory mutations in helices 3, 9, 13 and 14 render the enzyme competent to regulate the proton conduction through the proton channel. The sequence information of the *Porphyromonas* strain is less complete which makes an analysis with respect to compensatory mutations difficult. Finally, it should be stressed that, before any far reaching conclusions regarding the role of βAsn91 in *Mycobacterium* and *Porphyromonas* transhydrogenases are made, the functional properties of these enzymes with regard to proton pumping must be characterised.

5. Mechanism of communication between dII and dIII

There is presently no experimental evidence supporting the possibility that the proton pathway extends into dIII and the NADP(H)-binding site. Even though this is still an open question it is assumed, as a working hypothesis, that the protons exit immediately after crossing dII.

Several pieces of evidence strongly suggest that the peptide connecting dII and dIII is responsible for the communication between βHis91 and the NADP(H)-binding site. Thus, the trypsin sensitivity of the β subunit of *E. coli* transhydrogenase is dependent on the presence of NADP(H), i.e., the substrate triggers a conformational change that exposes the bond between βR265 and βS266, which is cleaved by the protease [33]. This characteristic feature is not dependent on interactions with dI, since the same cleavage occurs upon addition of NADP(H) even after digestion of dI. Note that βR265 is only five residues from

the C-terminal end of the transmembrane helix predicted as being the last one. In addition, the reactivity of cysteine residues in the βR265 with thiol reagents is strongly affected by the presence of absence of NADP(H). Furthermore, mutation of the βR265 residue strongly affected transhydrogenase activities. (M. Axelsson and J. Rydström, unpublished). These results strongly suggest that the communication between dII and dIII is mediated through a hinge region between β260 and approx. 290. It is thus very likely that the structural changes caused by NADP(H) binding are not confined to the binding pocket but propagate to dII as well. They could, for example, move dIII in closer contact with the membrane and the proton channel. This view is supported by the resistance of βM214C to modification by fluorescein maleimide in the presence but not in the absence of NADP(H) (J. Meuller, unpublished). Indeed, a communication between the NADP(H) site and domain II has been validated by several observations. For example, the β subunit in the mutants βH91C, βH91S and βH91T was no longer digestible by trypsin in the presence or in the absence of NADP(H). In contrast, the βH91K mutant was trypsin sensitive even in the absence of added NADP(H) probably due to substoichiometric amounts of tightly bound $NADP^+$. Also, a mutant lacking the six C-terminal residues in the α subunit contained $NADP^+$ and was cleaved by trypsin [6,30].

Based partly on the recently solved crystal structures of dIII from bovine [20] and human [21,22], but mainly on prediction, it was hypothesised that the 'acidic patch' [20] and the region with helix D and loop D [21,22] constituted the dII-dIII interface. It was also suggested that the 'acidic patch' interacted with dI [21,22]. Recent NMR experiments indicate, however, that dI does not interact with the 'acidic patch' (A. Bergkvist, C. Johansson, T. Johansson, J. Rydström and B.K. Karlsson, unpublished), suggesting that this region instead interacts with dII. The identity and function of these surfaces are presently being investigated.

Acknowledgements

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